

# Phylogenetic Diversity and Microsphere Array-Based Genotyping of Human Pathogenic *Fusaria*, Including Isolates from the Multistate Contact Lens-Associated U.S. Keratitis Outbreaks of 2005 and 2006<sup>∇‡</sup>

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**In 2005 and 2006, outbreaks of *Fusarium* keratitis associated with soft contact lens use occurred in multiple U.S. states and Puerto Rico. A case-control study conducted by the Centers for Disease Control and Prevention (CDC) showed a significant association between infections and the use of one particular brand of lens solution. To characterize the full spectrum of the causal agents involved and their potential sources, partial DNA sequences from three loci (*RPB2*, *EF-1α*, and nuclear ribosomal rRNA) totaling 3.48 kb were obtained from 91 corneal and 100 isolates from the patient's environment (e.g., contact lens and lens cases). We also sequenced a 1.8-kb region encoding the RNA polymerase II second largest subunit (*RPB2*) from 126 additional pathogenic isolates to better understand how the keratitis outbreak isolates fit within the full phylogenetic spectrum of clinically important *Fusaria*. These analyses resulted in the most robust phylogenetic framework for *Fusarium* to date. In addition, *RPB2* nucleotide variation within a 72-isolate panel was used to design 34 allele-specific probes to identify representatives of all medically important species complexes and 10 of the most important human pathogenic *Fusarium* in a single-well diagnostic assay, using flow cytometry and fluorescent microsphere technology. The multilocus data revealed that one haplotype from each of the three most common species comprised 55% of CDC's corneal and environmental isolates and that the corneal isolates comprised 29 haplotypes distributed among 16 species. The high degree of phylogenetic diversity represented among the corneal isolates is consistent with multiple sources of contamination.**

*Fusarium* keratitis (FK) is a rare sight-threatening corneal infection among the many contact lens wearers in the United States and abroad (5, 20). After an increase in FK among contact lens wearers was suspected in Singapore, an investigation in that country suggested a link with the use of ReNu brand contact lens solution (20), based on the disproportionate number of FK cases associated with this product. After an increase in cases of FK was suspected in the United States, the Centers for Disease Control and Prevention (CDC) in conjunction with other public health agencies in the United States conducted a case-control study, which demonstrated that the FK outbreaks in the United States were associated with use of Bausch & Lomb's ReNu with MoistureLoc (Rochester, NY) brand of contact lens solution (5). After Bausch & Lomb

recalled and then permanently discontinued worldwide sales of this product, the number of FK cases decreased substantially in Singapore and the United States (5, 20). Since corneal infections caused by *Fusarium* are typically associated with ocular trauma with soil, organic, or plant matter (5), the outbreaks, which involved cases of FK unassociated with ocular trauma, were unusual.

To date, microbiological assessment of the *Fusaria* associated with the outbreaks has involved DNA typing using single (20) and multilocus DNA sequence data (5, 9), mini- and microsatellite primer probes (9), and phenotypic characterization of cultures obtained from corneal scrapings (1). The latter study also reported on a preliminary assessment of *Fusaria* recovered from patients' contact lenses and lens cases.

Accurate identification of the etiological agents causing mycotic infections is critical to advancing our understanding of the environmental reservoir of each *Fusarium* species and may provide guidance to the most efficacious regimen of antifungal therapy. This is particularly important for *Fusaria*, for which antifungal MICs are typically higher and more variable than for human pathogenic aspergilli (3). Even though the great majority of *Fusaria* pathogenic to humans and other animals are nested within either the *Fusarium solani* or the *F. oxysporum* species complex (38), it has been traditional taxonomic practice to refer to members of these complexes as single

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species (i.e., *F. solani* and *F. oxysporum*). However, recent molecular phylogenetic studies have begun to clarify species boundaries within evolutionary lineages of *Fusarium* that contain human pathogens (33, 39, 44). Similar investigations of species limits within the *Gibberella fujikuroi* species complex have also been conducted (30, 32). A clear consensus that has emerged from these studies is that DNA sequence-based methods will be essential for species identification and subtyping of fusaria in clinical laboratories, especially within the pathogen-rich *Fusarium solani* species complex (FSSC) (5, 44). Indeed, the proactive DNA sequence characterization of human pathogenic isolates from these complexes, derived from major fungal culture collections, greatly assisted the rapid and precise identification of the pathogens involved in the keratitis outbreaks of 2005 and 2006 and subsequent epidemiological inferences (5). Zhang et al. (44) found that all of the 278 *F. solani* complex isolates from humans were members of a previously defined major clade within the complex ("clade 3") (29), with approximately three-quarters of them falling into four major groups designated FSSC groups 1 to 4. These researchers also found that isolates from eye infections spanned the phylogenetic breadth of clade 3, which comprises approximately 25 phylogenetic species, with a statistically significant association with FSSC group 3 that was otherwise associated with soil, plants, and plant debris. Fortunately, recent advances in the development of allele-specific assays for identification of human pathogenic fungi (6, 35) and bacterial subtyping (8) clearly demonstrate that fluorescent microsphere array technology offers a powerful platform for rapid and accurate pathogen typing required by the public health community to respond to outbreaks in a timely manner.

Therefore, the objectives of the present study were threefold: (i) to investigate the full spectrum of fusaria associated with the keratitis outbreaks within the United States (including Puerto Rico), we examined the phylogenetic diversity of 91 corneal and 100 environmental isolates associated with the outbreaks, using DNA sequence data from three loci (5); (ii) to assess how the keratitis outbreak isolates fell within the full phylogenetic spectrum of *Fusarium*, nucleotide sequence data from the RNA polymerase II second largest subunit (*RPB2*) was used for the first time to identify medically important species and evolutionary lineages (i.e., monophyletic species complexes) and to infer a robust phylogenetic structure for *Fusarium*; and (iii) to develop and validate a high throughput, microsphere array for the simultaneous detection and identification of human pathogenic fusaria in a single well using flow cytometry, *RPB2* nucleotide sequence data was used to design allele-specific probes for the six medically important species complexes and ten of the most important human pathogenic species. In contrast to CDC's published case-control study (5), in which only confirmed case isolates were included, we have analyzed here the full spectrum of isolates from patient corneas and their environment acquired for study by the CDC during the outbreak investigation (Table 1 and Table S1 in the supplemental material).

#### MATERIALS AND METHODS

**Fungal strains.** The 319 isolates included in the present study were analyzed as the following three groups: a panel of 191 CDC keratitis outbreak isolates (Table 1 and Table S1 in the supplemental material) that were genotyped using

multilocus DNA sequence data as described previously (5), a panel of 72 isolates for the design and validation of the microsphere array (design panel [DP]) (Table 2), and an experimental panel (EP) comprising 157 additional isolates (Table 3). In addition, an *RPB2* sequence obtained from *Lecanicillium lecanii*, a member of *Clavicipitaceae* in the *Hypocreales*, was chosen as an outgroup for the phylogenetic analysis of the design panel data set (Fig. 1) based on more inclusive analyses (23). The DP and EP contained 36 and 69 outbreak isolates, respectively. All of the outbreak investigation isolates obtained from corneal scrapings, and the environmental assessment were verified as fusaria morphologically at the CDC (12, 27). In addition to the keratitis outbreak isolates, 128 pathogenic isolates were obtained from various sources for genotyping (Tables 2 and 3), including 54 phylogenetically diverse fusaria from The University of Texas Health Science Center's Fungus Testing Laboratory, San Antonio, TX. All strains are stored cryogenically and are available upon request from the Agricultural Research Service (NRRL) Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL.

**DNA isolation and PCR amplification for multilocus DNA sequencing.** Fungal mycelium was grown in yeast-malt broth (20 g of dextrose, 5 g peptone, 3 g of yeast extract, and 3 g of malt extract per liter; Difco, Detroit, MI) and freeze-dried, and then a hexadecyltrimethyl-ammonium bromide (CTAB; Sigma, St. Louis, MO) protocol was used to extract total genomic DNA as described previously (30). Portions of the nuclear large rRNA subunit (LSU) and translation elongation factor (*EF-1 $\alpha$* ) genes were amplified by PCR as described previously (5). Two contiguous regions of the RNA polymerase II second largest subunit (*RPB2*) were amplified with the PCR primers 5F2 (5'-GGGGWGAYCAGAAGAAGGC) and 7cR (5'-CCCATRGCTTGYYTRCCCAT) and the primers 7cF (5'-ATGGGYAARCAAGCYATGGG) and 11aR (5'-GCRTGGATCTTRTCRTCSACC) in separate reactions (36), using Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) in an Applied Biosystems 9700 thermocycler (Emeryville, CA), and by following a PCR program of 1 cycle of 90 s at 94°C, followed by 40 cycles of 30 s at 94°C, 90 s at 55°C, and 2 min at 68°C, followed in turn by 1 cycle of 5 min at 68°C and a 4°C soak. After agarose electrophoresis and ethidium bromide staining, the amplicons were visualized over a UV transilluminator, and then they were purified by using Montage<sub>66</sub> filter plates (Millipore Corp., Billerica, MA). Amplicons were sequenced as described in O'Donnell et al. (33). Raw sequence data were edited and aligned by using Sequencer version 4.1.2 (Gene Codes, Ann Arbor, MI) and then manipulated manually to establish positional homology.

**Phylogenetic analysis.** Initially, the neighbor-joining method, using Kimura's two-parameter model implemented in PAUP\* (40), was used to assess the phylogenetic diversity of *RPB2* sequences of all 319 isolates included in the present study. Based on these analyses, we constructed a DP (Table 2) data set, which included aligned sequences from 72 fusaria plus the outgroup sequence of *Lecanicillium lecanii*, to further investigate the phylogenetic diversity of medically important fusaria. One indel, 3 bp in length, was inserted in the *RPB2* alignment to accommodate for one additional codon unique to all members of the *Fusarium dimerum* species complex (FDSC). A second indel, 39 bp in length, was inserted in the alignment to adjust for 13 additional amino acids present in all members of the *Gibberella* clade. A parsimony search of the DP (Table 2) data set conducted with PAUP\* version 4.0b10 (40) was implemented, using tree-bisection and reconnection branch swapping and 1,000 random sequence addition replicates. Only parsimony-informative nucleotide positions were included in the analysis. Nonparametric bootstrapping was used to assess clade stability and relative support for internodes, based on 1,000 pseudoreplicates of the data. One of the 186 most-parsimonious trees (MPTs) found from the maximum-parsimony heuristic search was saved for use in the design of allele-specific probes.

**Allele-specific probe design.** Character state changes identified in the DNA sequence were mapped on the MPT in order to identify candidate allele-specific probes, using MacClade's data editor (24). Aligned *RPB2* sequences of the DP were used to develop 34 allele-specific probes for all six medically important species complexes (15 probes), ten of the most important species (15 probes), plus 4 probes designed for use as positive controls for the PCR amplification (Table 4 and Fig. 1). Assignment of isolates to one unnamed species within the FSSC, referred to as FSSC group 4 (44), was based on positive and negative genotypes for the S-34 and S-4 probes, respectively. The oligonucleotide probes range in length from 18 to 24 nucleotides (Table 4), and every probe ends with a 3' single nucleotide difference specific to a species and/or species complex, except for the four positive control probes that represent sequences highly conserved throughout the genus. Once designed, all 34 probes were submitted to the Luminex Corp. website (Austin, TX), where they were assigned a unique 24-bp sequence tag, which was incorporated on the 5' end of each probe primer (Table 4). During the hybridization step (see below), each unique sequence

TABLE 1. Summary of CDC keratitis investigation isolates<sup>a</sup>

Complex	Species <sup>b</sup>	Haplotype <sup>c</sup>	No. of isolates (%) <sup>d</sup>		
			Corneal	Environmental	Combined
FSSC	FSSC-1	1-a	54 (59.3)	64 (64)	118 (61.8)
		1-b	13 (18)	24 (27)	37 (45)
		1-c	3	2	5
		1-d	2 (4)	2 (3)	4 (7)
		1-e	1	0	1
	FSSC-2	2-a	2 (4)	0	2 (4)
		2-b	2	0	2
		2-d	9 (13)	11 (14)	21 (27)
		2-f	0	2 (4)	2 (4)
		2-g	0	1	1
	FSSC-3	2-h	1	0	1
		2-i	0	1	1
		2-j	1	0	1
		3-a	0	2 (4)	2 (4)
		3-b	2	0	2
	FSSC-4	3-c	1	0	1
		4-a	1	0	1
	FSSC-5	4-b	1	0	1
		5-a	0	1	1
	FSSC-6	5-b	0	1	1
		5-c	1	0	1
		5-d	0	1 (3)	1 (3)
	FSSC-7	6-a	0	1	1
	FSSC-8	7-a	0	1	1
	FSSC-9	8-a	1	0	1
		9-a	0	1	1
FOSC	FOSC-3	3-a	27 (29.7)	29 (29)	56 (29.3)
		3-b	10 (15)	16 (19)	26 (34)
		3-c	0	1	1
		3-d	1	0	1
		3-e	0	1	1
		3-f	1 (2)	2	3 (4)
		3-g	1	0	1
	FOSC-4	2 (4)	1	1	2
		4-a	1	0	1
		4-b	2 (3)	1	3 (4)
GFSC		4-c	0	1 (4)	1 (4)
			6 (6.6)	6 (6)	12 (6.3)
	<i>F. fujikuroi</i>		0	1	1
	<i>F. proliferatum</i>		1 (2)	1	2 (3)
	<i>F. sacchari</i>		0	1	1
	<i>F. thapsinum</i>		1	0	1
	<i>F. verticillioides</i>		1	1	2
FIESC	<i>F. sp. cf. verticillioides</i>		1 (2)	2	3 (4)
			2 (2.2)	2 (2%)	4 (2.1)
	<i>F. sp. cf. incarnatum-1</i>	1-a	0	1	1
	<i>F. sp. cf. incarnatum-2</i>	2-a	1	0	1
	<i>F. sp. cf. incarnatum-3</i>	3-a	1	0	1
FDSC	<i>F. sp. cf. incarnatum-4</i>	4-a	0	1	1
			1 (1.1)	0	1 (0.5)
	<i>F. sp. cf. dimerum</i>	1-a	1	0	1
Total			67 (91)	81 (100)	148 (191)

<sup>a</sup> See Table S1 in the supplemental material for the histories of all 191 isolates.<sup>b</sup> Species names can only be applied with confidence to five species with in the GFSC (30, 32). The remaining 17 unnamed species are distributed among the following species complexes: 9 with in the FSSC (1 to 9), 2 with in the FOSSC (3 and 4), *F. sp. cf. verticillioides* with in the GFSC, 4 with in the FIESC (*F. sp. cf. incarnatum* 1 to 4), and *F. sp. cf. dimerum* with in the FDSC.<sup>c</sup> Haplotypes were determined by multilocus DNA sequence-based genotyping as described in Materials and Methods.<sup>d</sup> The number of isolates in parentheses for each species studied by MLST includes putative clones (see Table S1 in the supplemental material for details). The frequency (%) of isolates recovered from cornea and the patient's environment for each species complex includes putative clones.

TABLE 2. Design panel of fusaria analyzed by allele-specific genotyping and *RPB2* sequencing

NRRL no.	Equivalent no. <sup>a</sup>	Yr	Isolate source	Origin	Identified by Luminex/RPB2 <sup>b</sup>	ID <sup>c</sup> (complex/species probe) <sup>d</sup>
3211	Sandoz 484	1966		Switzerland	FIESC	10.7 (IEC-f)
5537	ATCC 28805		Fescue hay	Missouri	FIESC	4.6 (IEC-f)
13335	FRC R-2138				FIESC	11.5 (IEC-f)
13379	FRC R-5198				FIESC	14.6 (IEC-f)
13448	CBS 749.97	1997	Sorgum root	New South Wales, Australia	GFSC/F. <i>nygamai</i>	4.1 (GFC-c)
13566	IMI 375349		Rice	Taiwan	<i>F. fujikuroi</i>	3.9 (GFC-c), 13.7 (F-c)
13604	CBS 748.97	1997	Millet	Namibia	GFSC/F. <i>napiiforme</i>	2.9 (GFC-c)
13614	CBS 258.54	1954	Rice (NRRL 13614)	Vietnam	<i>F. proliferatum</i>	4.8 (GFC-c), 16.6 (P-a)
13713	FRC T-428		Millet	South Africa	FCSC	10.7 (CC-c)
20423	ATCC 42771		Lizard skin	India	FIESC	19.7 (IEC-f)
20425	CBS 131.73	1973	Banana	Bahamas	FIESC	11.8 (IEC-f)
22172	CBS 734.97	1997	Corn	Germany	<i>F. verticillioide</i>	3.1 (GFC-c), 9.4 (V-a)
25229	IMI 240460		Mycetoma	Italy	<i>F. thapsinum</i>	5.4 (GFC-c), 11.4 (T-e)
25483	CBS 217.78	1978	Millet	Namibia	FCSC	14.8 (CC-c)
25489	CBS 144.44	1944			FIESC	5.9 (IEC-f)
26360	FRC O-755		Cornea	Tennessee	FOSC	3.7 (OC-a)
26421	CBS 140.95	1995	Systemic infection	Egypt	GFSC/F. <i>nygamai</i>	7.1 (GFC-c)
28023	CBS 537.81		Coccidia	Galapagos Islands	<i>Lecanicillium lecanii</i>	N/A
28505	FRC R-8670		Soil debris	South Africa	FCSC	8.7 (CC-c)
28682	96-6008			Germany	<i>F. verticillioide</i>	4.4 (GFC-c), 14.6 (V-a)
31005	BBA 62211			Iran	FIESC	8.4 (IEC-f)
31010	BBA 64316		<i>Artemisia vulgaris</i>	Germany	FIESC	16.8 (IEC-f)
31011	BBA 69079		<i>Thuja</i>	Germany	FIESC	10.9 (IEC-f)
31160	MDA #3		Lung	Texas	FIESC	3.9 (IEC-f)
31167	MDA #10		Sputum, leukemia	Texas	FIESC	11.9 (IEC-f)
32173	MDA F2		Arm tissue, leukemic	Delaware	<i>F. sp. cf. dimerum</i>	11.0 (DC-d), 5.6 (D-c)
32174	MDA F8		Skin, leukemic	Florida	<i>F. proliferatum</i>	4.1 (GFC-c), 16.2 (P-a)
32175	MDA F10		Sputum	Texas	FIESC	10.7 (IEC-f)
32179	MDA F18		Sputum, leukemic	Texas	<i>F. thapsinum</i>	4.6 (GFC-c), 11.3 (T-e)
32434	CBS 623.92	1992	Foot lesion	Germany	FSSC	6.8 (SC-d)
32521	FRC T-852		Cancer patient		FCSC	10.9 (CC-c)
32868	FRC R-8880		Blood	Ohio	FIESC	11.0 (IEC-f)
34032	UTHSC 98-2172	1998	Mandibular abscess	Texas	FIESC	6.1 (IEC-f)
36064	FRC O-1747				FOSC	6.2 (OC-a)
36168	CBS 110148		<i>Gymnocalycium damsii</i>	Germany	FDSC	35.1 (DC-d)
36511	CBS 572.94	1994	Pigeon pea	India	GFSC/F. <i>nygamai</i>	6.1 (GFC-c)
36536	CBS 673.94	1994			GFSC/F. <i>napiiforme</i>	4.5 (GFC-c)
43373	CDC 2006743215	2006	Contact lens	Malaysia	FSSC 2	2.6 (SC-d), 4.5 (S-2)
43374	CDC 2006743216	2006	Cornea	Nigeria	FSSC 2	12.1 (SC-d), 18.4 (S-2)
43375	CDC 2006011016	2006	Cornea	New Jersey	FSSC 1	8.5 (SC-d), 4.2 (S-1)
43431	CDC 2006011126	2006	Cornea	Connecticut	FOSC	5.7 (OC-a)
43433	CDC 2006011214	2006	Cornea	Ohio	FSSC 2	20.2 (SC-d), 29.7 (S-2)
43441	CDC 2006743414	2006	Cornea	Pennsylvania	FSSC 3	10.6 (SC-d), 11.9 (S-34)
43442	CDC 2006743415	2006	Cornea	Pennsylvania	FOSC	5.0 (OC-a)
43445	CDC 2006743419	2006	Cornea	Connecticut	FSSC 2	15.1 (SC-d), 22.0 (S-2)
43454	CDC 2006743427	2006	Cornea	Massachusetts	FOSC	6.0 (OC-a)
43455	CDC 2006743230	2006	Cornea	Vermont	FOSC	5.1 (OC-a)
43458	CDC 2006743233	2006	Cornea	Singapore	FSSC 2	14.3 (SC-d), 23.0 (S-2)
43464	CDC 2006743239	2006	Cornea	Singapore	FSSC 2	13.8 (SC-d), 21.8 (S-2)
43466	CDC 2006743429	2006	Contact lens case	Ohio	FOSC	4.9 (OC-a)
43468	CDC 2006743431	2006	Cornea	Iowa	FSSC	4.8 (SC-d)
43470	CDC 2006743433	2006	Cornea	Illinois	<i>F. fujikuroi</i>	2.7 (GFC-c), 10.5 (F-c)
43474	CDC 2006743441	2006	Cornea	Illinois	FSSC	8.7 (SC-d)
43489	CDC 2006743456	2006	Cornea	Maryland	FSSC	7.4 (SC-d)
43490	CDC 2006743457	2006	Cornea	Michigan	FSSC 2	6.4 (SC-d), 8.7 (S-2)
43503	CDC 2006743471	2006	Contact lens case	Connecticut	FSSC 4	11.3 (SC-d), 11.6 (S-4)
43504	CDC 2006743483	2006	Cornea	Pennsylvania	FOSC	5.8 (OC-a)
43514	CDC 2006743560	2006	Cornea	Florida	FSSC 2	11.9 (SC-d), 15.9 (S-2)
43521	CDC 2006743567	2006	Cornea	Florida	FOSC	6.3 (OC-a)
43527	CDC 2006743573	2006	Cornea	Florida	FSSC	3.8 (SC-d)
43528	CDC 2006743574	2006	Cornea	Florida	FSSC 2	7.1 (SC-d), 12.1 (S-2)
43529	CDC 2006743575	2006	Cornea	Florida	FSSC 4	7.4 (SC-d), 7.4 (S-4)
43532	CDC 2006743578	2006	Cornea	Florida	FSSC 2	15.6 (SC-d), 20.8 (S-2)
43533	CDC 2006743579	2006	Cornea	Florida	FSSC 1	5.9 (SC-d), 3.4 (S-1)
43536	CDC 2006743582	2006	Cornea	Florida	FSSC 3	6.4 (SC-d), 8.1 (S-34)
43537	CDC 2006743583	2006	Cornea	Florida	FSSC 3	9.7 (SC-d), 11.5 (S-34)
43539	CDC 2006743490	2006	Cornea	Missouri	FOSC	6.9 (OC-a)
43540	CDC 2006743491	2006	Cornea	Kansas	FSSC 1	6.1 (SC-d), 2.8 (S-1)
43655	CDC 2006743515	2006	Contact lens case fluid	Ohio	FOSC	5.9 (OC-a)
43656	CDC 2006743516	2006	Contact lens	Minnesota	<i>F. verticillioide</i>	2.8 (GFC-c), 11.4 (V-a)
43665	CDC 2006743525	2006	Contact lens	North Carolina	<i>F. proliferatum</i>	3.4 (GFC-c), 12.7 (P-a)
43668	CDC 2006743529	2006	Cornea	New Jersey	FOSC	6.1 (OC-a)
43726	CDC 2006743692	2006	Cornea	Ohio	FDSC	53.2 (DC-d)

<sup>a</sup> ATCC, American Type Culture Collection, Manassas, VA; BBA, Biologische Bundesanstalt für Land- und Forstwirtschaft, Institute für Mikrobiologie, Berlin, Germany; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CDC, Centers for Disease Control and Prevention, Atlanta, GA; FRC, Fusarium Research Center, The Pennsylvania State University, State College, PA; IMI, CABI Biosciences, Egham, Surrey, England; MDA, M. D. Anderson Cancer Center, Houston, TX; UTHSC, University of Texas Health Sciences Center, San Antonio, TX.

<sup>b</sup> Isolates belonging to FSSC groups 1 to 4 are indicated (5, 44).

<sup>c</sup> ID, index of discrimination determined as minimum target fluorescent intensity/maximum nontarget fluorescence intensity.

<sup>d</sup> See Table 4 for a complete listing of the probes. Note that the probes listed here lack the F for *Fusarium* prefix.



TABLE 3. Experimental panel of fusaria analyzed by allele-specific genotyping and *RPB2* sequencing

NRRL no.	Equivalent no. <sup>a</sup>	Yr	Isolate source	Origin	Identified by Luminex/RPB2 <sup>b</sup>	ID <sup>c</sup> (complex/species probe <sup>d</sup> )
13459	CBS 961.87	1984	Plant debris	South Africa	<i>F. proliferatum</i> / <i>F. concolor</i>	0 (GFC-c, GFCO-c), 6.7 (PF-b)
28000	CDC B-4536	1987	Blood, leukemic	Georgia	FSSC 3/FSSC	23.5 (SC-d), 17.0 (S-34)
28001	CDC B-4572	1987	Skin, leukemic	Colorado	FSSC 3/FSSC	33.1 (SC-d), 47.1 (S-34)
28002	CDC B-4581	1987	Skin, leukemic	Georgia	FSSC 3/FSSC	29.6 (SC-d), 19.6 (S-34)
28003	CDC B-4680	1988	Toenail	Washington, DC	<i>F. fujikuroi</i>	4.4 (GFC-a), 14.6 (F-c)
28005	CDC B-4687	1988	Hospital venilation system	Illinois	<i>F. proliferatum</i>	8.9 (GFC-c), 17.0 (P-a)
28006	CDC B-4688	1988	Hospital venilation system	Illinois	<i>F. thapsinum</i>	3.3 (GFC-c), 6.2 (T-e)
28007	CDC B-4689	1988	Hospital venilation system	Illinois	<i>F. thapsinum</i>	3.5 (GFC-c), 8.9 (T-e)
28008	CDC B-4701	1988	Hospital venilation system	Illinois	FSSC 1/FSSC	5.9 (SC-d), 7.2 (S-1)
28009	CDC B-5543	1994	Vitrous fluid	Texas	FSSC 1/FSSC	25.2 (SC-d), 6.5 (S-1)
28010	CDC B-5546	1995	Vitrous fluid	Texas	FSSC 1/FSSC	36.4 (SC-d), 7.1 (S-1)
28011	CDC B-5548	1994	Vitrous fluid	Texas	FSSC 1/FSSC	29.3 (SC-d), 7.0 (S-1)
28014	CDC B-5754	1997	Leg wound, diabetic	California	FSSC 2	9.8 (SC-d), 13.5 (S-2)
28016	CDC B-5779	1997	Bronchial wash, leukemic	Ohio	FSSC	7.1 (SC-d)
28017	CDC B-5780	1997	Catheter, leukemic	Ohio	FSSC	19.1 (SC-d)
28018	CDC B-5781	1997	Catheter, leukemic	Ohio	FSSC	8.2 (SC-d)
28019	CDC B-5782	1997	Skin lesion, leukemic	Ohio	FSSC	17.5 (SC-d)
28029	CDC B-3335	1980	Corneal transplant	California	FIESC	9.6 (IEC-f)
28030	CDC B-3723	1983	Skin lesion, leukemic	Thailand	FSSC	4.6 (SC-d)
28031	CDC B-3882	1984	Toe nail	South Carolina	FOSC	7.1 (OC-a)
28033	CDC B-4422	1987	Leukemic	Italy	FSSC 1	14.5 (SC-d), 5.9 (S-1)
28035	CDC B-4809	1989	Lymph node	Arkansas	<i>F. proliferatum</i>	3.5 (GFC-c), 8.4 (P-a)
37068	CBS 110192		Soil	Australia	FDSC	5.4 (DC-d)
37071	CBS 110320		Toenail	Chile	<i>F. sp. cf. dimerum</i>	15.9 (DC-d), 7.4 (D-c)
43544	CDC 2006743496	2006	Cornea	Ohio	FSSC 1	13.9 (SC-d), 7.4 (S-1)
43545	CDC 2006743497	2006	Cornea	Ohio	<i>F. proliferatum</i>	4.0 (GFC-c), 15.2 (P-a)
43547	CDC 2006743499	2006	Contact lens	Minnesota	<i>F. verticillioide</i> s	2.6 (GFC-c), 11.9 (V-a)
43583	UTHSC 06-989	2006	Cornea	Ohio	FSSC 1	13.3 (SC-d), 5.8 (S-1)
43584	UTHSC 06-743	2006	Catheter tip	Florida	FSSC 3	6.2 (SC-d), 6.9 (S-34)
43585	UTHSC 06-295	2006	Tissue	Utah	FSSC 2	3.2 (SC-d), 5.8 (S-2)
43586	UTHSC 05-3538	2005	Cornea	Texas	FSSC 2	11.1 (SC-d), 18.8 (S-2)
43588	UTHSC 05-3530	2005	Corneal transplant	Illinois	FSSC 1	17.1 (SC-d), 16.4 (S-1)
43589	UTHSC 05-3333	2005	Blood	Ohio	FSSC 1	13.2 (SC-d), 5.2 (S-1)
43590	UTHSC 05-2824	2005	Blood	Maine	FSSC 1	14.1 (SC-d), 7.8 (S-1)
43591	UTHSC 05-2680	2005	Cornea	Florida	FSSC 1	15.7 (SC-d), 7.1 (S-1)
43592	UTHSC 06-689	2006	Cornea	Massachusetts	FOSC	5.7 (OC-a)
43593	UTHSC 05-3159	2005	Nail	South Carolina	FOSC	6.7 (OC-a)
43594	UTHSC 05-1643	2005	Cornea	Massachusetts	FOSC	5.7 (OC-a)
43595	UTHSC 05-721	2005	Blood	California	FOSC	4.0 (OC-a)
43596	UTHSC 04-1934	2004	Lung	Arkansas	FOSC	5.7 (OC-a)
43597	UTHSC 04-584	2004	Bronchial wash	Utah	FOSC	4.5 (OC-a)
43598	UTHSC 03-753	2003	Foot tissue	Florida	FOSC	5.4 (OC-a)
43599	UTHSC 06-1103	2006	Toe	Florida	<i>F. verticillioide</i> s	17.2 (GFC-c), 4.7 (V-a)
43600	UTHSC 05-3141	2005	Cornea	Massachusetts	<i>F. verticillioide</i> s	6.1 (GFC-c), 25.1 (V-a)
43601	UTHSC 05-1039	2005	Skin	Maryland	<i>F. verticillioide</i> s	5.0 (GFC-c), 29.0 (V-a)
43602	UTHSC 05-850	2005	Sputum	Wisconsin	<i>F. verticillioide</i> s	7.1 (GFC-c), 26.3 (V-a)
43603	UTHSC 05-431	2005	Skin	Ohio	<i>F. verticillioide</i> s	6.5 (GFC-c), 22.5 (V-a)
43604	UTHSC 05-430	2005	Nasal sinus	Ohio	<i>F. verticillioide</i> s	5.6 (GFC-c), 26.3 (V-a)
43605	UTHSC 04-700	2004	Skin	Massachusetts	<i>F. verticillioide</i> s	6.6 (GFC-c), 25.0 (V-a)
43606	UTHSC 04-695	2004	Trachea	Louisiana	<i>F. verticillioide</i> s	5.9 (GFC-c), 25.3 (V-a)
43608	UTHSC 03-2552	2003	Peritoneal fluid	Minnesota	<i>F. verticillioide</i> s	4.5 (GFC-c), 15.4 (V-a)
43609	UTHSC 06-952	2006	Palate	Florida	<i>F. proliferatum</i>	5.6 (GFC-c), 19.2 (P-a)
43610	UTHSC 06-836	2006	Skin	Iowa	<i>F. fujikuroi</i>	15.9 (GFC-c), 17.0 (F-c)
43611	UTHSC 06-492	2006	Lacrimal duct	Texas	<i>F. proliferatum</i>	2.7 (GFC-c), 9.4 (P-a)
43612	UTHSC 06-432	2006	Nail	Florida	<i>F. fujikuroi</i>	4.5 (GFC-c), 15.1 (F-c)
43613	UTHSC 06-197	2006	Cornea	Utah	<i>F. proliferatum</i>	5.3 (GFC-c), 19.8 (P-a)
43614	UTHSC 04-1974	2004	Nose	Texas	<i>F. proliferatum</i>	6.0 (GFC-c), 22.9 (P-a)
43615	UTHSC 04-1772	2004	Ethmoid sinus	Ohio	<i>F. proliferatum</i>	6.9 (GFC-c), 23.7 (P-a)
43616	UTHSC 03-3232	2003	Tissue	Utah	<i>F. proliferatum</i>	6.0 (GFC-c), 21.8 (P-a)
43617	UTHSC 03-60	2003	Blood	Colorado	<i>F. proliferatum</i>	7.2 (GFC-c), 25.0 (P-a)
43618	UTHSC 02-1631	2002	Blood	Texas	<i>F. proliferatum</i>	3.8 (GFC-c), 13.0 (P-a)
43619	UTHSC 05-2847	2005	Index finger	Texas	FIESC	12.7 (IEC-f)

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TABLE 3—Continued

NRRL no.	Equivalent no. <sup>a</sup>	Yr	Isolate source	Origin	Identified by Luminex/RPB2 <sup>b</sup>	ID <sup>c</sup> (complex/species probe <sup>d</sup> )
43622	UTHSC 03-2501	2003	Lung	Texas	FIESC	8.5 (IEC-f)
43623	UTHSC 03-59	2003	Maxillary sinus	Colorado	FIESC	18.7 (IEC-f)
43624	UTHSC 02-2060	2002	Sputum	Texas	FIESC	11.8 (IEC-f)
43625	UTHSC 02-1698	2002	Maxillary sinus	Texas	FIESC	12.8 (IEC-f)
43627	UTHSC 05-3559	2005	Bronchial wash	Texas	FCSC	15.5 (CC-c)
43628	UTHSC 05-3396	2005	Finger wound	Florida	FCSC	16.1 (CC-c)
43629	UTHSC 05-3200	2005	Blood	Utah	FCSC	14.7 (CC-c)
43630	UTHSC 05-2743	2005	Sputum	Texas	FCSC	5.0 (CC-c)
43631	UTHSC 05-2441	2005	Leg biopsy	Texas	FCSC	18.7 (CC-c)
43632	UTHSC 05-1260	2005	Cornea	Florida	FCSC	10.0 (CC-c)
43633	UTHSC 03-3472	2003	Maxillary sinus	Tennessee	FCSC	9.6 (CC-c)
43634	UTHSC 02-1276	2002	Horse cornea	Alabama	FCSC	22.2 (CC-c)
43635	UTHSC 06-638	2006	Horse	Nebraska	FIESC	12.9 (IEC-b)
43636	UTHSC 06-170	2006	Dog	Texas	FIESC	13.3 (IEC-f)
43637	UTHSC 05-1729	2005	Dog, cutaneous	Pennsylvania	FIESC	20.1 (IEC-f)
43638	UTHSC R-3500	2004	Manatee	Florida	FIESC	9.0 (IEC-b)
43639	UTHSC 04-135	2004	Manatee	Florida	FIESC	13.2 (IEC-b)
43640	UTHSC 04-123	2004	Dog, nasal	Texas	FIESC	15.6 (IEC-f)
43641	UTHSC 06-1377	2006	Horse eye	Missouri	Unknown/ <i>Fusarium</i> sp.	2.9 (5f2-ab), 3.6 (7cf-ab)
43673	CDC 2006743501	2006	Contact lens case fluid	New Jersey	FSSC 2	11.2 (SC-d), 16.0 (S-2)
43674	CDC 2006743553	2006	Contact lens case	Kentucky	FOSC	3.7 (OC-a)
43675	CDC 2006743554	2006	Cornea	Kentucky	FSSC 1	14.4 (SC-d), 7.6 (S-1)
43676	CDC 2006743585	2006	Cornea	Illinois	FSSC 1	14.2 (SC-d), 7.9 (S-1)
43677	CDC 2006743586	2006	Contact lens case	Illinois	FSSC 1	3.9 (SC-d), 7.4 (S-1)
43678	CDC 2006743587	2006	Contact lens case	California	FOSC	4.0 (OC-a)
43679	CDC 2006743588	2006	Contact lens	California	FOSC	3.4 (OC-a)
43680	CDC 2006743589	2006	Contact lens case fluid	Texas	FIESC	10.9 (IEC-b)
43681	CDC 2006743591	2006	Cornea	South Dakota	FSSC	6.7 (SC-d)
43682	CDC 2006743592	2006	Cornea	Maine	<i>F. verticillioides</i>	2.6 (GFC-c), 11.5 (V-a)
43683	CDC 2006743593	2006	Cornea	California	FSSC 1	14.6 (SC-d), 8.4 (S-1)
43684	CDC 2006743594	2006	Cornea	California	FSSC 1	14.2 (SC-d), 8.3 (S-1)
43685	CDC 2006743595	2006	Cornea	California	FSSC 1	15.0 (SC-d), 5.9 (S-1)
43686	CDC 2006743597	2006	Contact lens case	California	FOSC	4.6 (OC-a)
43687	CDC 2006743599	2006	Contact lens solution cap	Kentucky	FOSC	4.8 (OC-a)
43690	CDC 2006743602	2006	Contact lens	Puerto Rico	FSSC 2	8.1 (SC-d), 13.5 (S-2)
43691	CDC 2006743603	2006	Contact lens	Puerto Rico	FSSC 2	6.4 (SC-d), 11.0 (S-2)
43692	CDC 2006743604	2006	Cornea	Washington	FOSC	3.3 (OC-a)
43693	CDC 2006743606	2006	Contact lens	Texas	<i>F. thapsinum</i>	4.5 (GFC-c), 12.0 (T-e)
43694	CDC 2006743607	2006	Cornea	Texas	FIESC	13.9 (IEC-f)
43695	CDC 2006743608	2006	Contact lens solution	Texas	FSSC 1	15.2 (SC-d), 10.0 (S-1)
43696	CDC 2006743609	2006	Cornea	Texas	FOSC	3.3 (OC-a)
43697	CDC 2006743611	2006	Cornea	Pennsylvania	<i>F. verticillioides</i>	2.9 (GFC-c), 12.8 (V-a)
43698	CDC 2006743612	2006	Cornea	Pennsylvania	FOSC	3.7 (OC-a)
43699	CDC 2006743615	2006	Contact lens case	Minnesota	FSSC 1	9.8 (SC-d), 6.0 (S-1)
43700	CDC 2006743616	2006	Cornea	Minnesota	FSSC 1	14.0 (SC-d), 8.0 (S-1)
43701	CDC 2006743617	2006	Cornea	Minnesota	FSSC 1	14.9 (SC-d), 8.0 (S-1)
43702	CDC 2006743618	2006	Contact lens case fluid	Ohio	FSSC 2	7.7 (SC-d), 13.7 (S-2)
43703	CDC 2006743619	2006	Contact lens case	South Dakota	FSSC	7.0 (SC-d)
43704	CDC 2006743620	2006	Contact lens	South Dakota	FSSC	5.9 (SC-d)
43705	CDC 2006743622	2006	Contact lens case fluid	Puerto Rico	FSSC 2	8.4 (SC-d), 13.3 (S-2)
43706	CDC 2006743623	2006	Cornea	Ohio	FOSC	3.9 (OC-a)
43707	CDC 2006743596	2006	Cornea	California	FOSC	4.2 (OC-a)
43708	CDC 2006743598	2006	Contact lens solution	California	FOSC	6.5 (OC-a)
43709	CDC 2006743600	2006	Contact lens case fluid	Kentucky	FOSC	5.9 (OC-a)
43710	CDC 2006743601	2006	Contact lens	Kentucky	FOSC	5.3 (OC-a)
43711	CDC IFI01-0048	2001	Sinus	Michigan	<i>F. proliferatum</i>	5.3 (GFC-c), 18.6 (P-a)
43713	CDC IFI02-0239	2002	Blood	North Carolina	FOSC	6.7 (OC-a)
43714	CDC IFI03-0214	2003	Sputum	Maryland	<i>F. verticillioides</i>	4.5 (GFC-c), 20.8 (V-a)
43715	CDC IFI04-0065	2004	Skin	Alabama	FSSC 1	5.6 (SC-d), 7.5 (S-1)
43716	CDC IFI04-0194	2004	Blood	-	<i>F. sp. cf. dimerum</i> /FDSC	10.9 (DC-d), 8.7 (D-b), 6.6 (D-c)
43717	CDC IFI03-0179	2003	SubQchest	Michigan	FSSC	9.2 (SC-d)
43724	CDC 2006743696	2006	Cornea	North Carolina	FSSC 1	12.3 (SC-d), 8.0 (S-1)
43725	CDC 2006743697	2006	Contact lens	North Carolina	FSSC 1	10.2 (SC-d), 8.7 (S-1)
43727	CDC 2006743694	2006	Cornea	Florida	FSSC 2	6.7 (SC-d), 12.7 (S-2)
43728	CDC 2006743691	2006	Cornea	Florida	FSSC 2	7.8 (SC-d), 13.1 (S-2)
43729	CDC 2006743621	2006	Contact lens case fluid	Puerto Rico	FSSC 2	8.5 (SC-d), 12.0 (S-2)
43730	CDC 2006743605	2006	Contact lens	Mississippi	FIESC	8.9 (IEC-f)

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TABLE 3—Continued

NRRL no.	Equivalent no. <sup>a</sup>	Yr	Isolate source	Origin	Identified by Luminex/RPB2 <sup>b</sup>	ID <sup>c</sup> (complex/species probe <sup>d</sup> )
43731	CDC 2006743689	2006	Contact lens case	Iowa	FSSC 1	8.9 (SC-d), 5.7 (S-1)
43732	CDC 2006743695	2006	Contact lens case	Michigan	FOSC	3.0 (OC-a)
43733	CDC 2006743623	2006	Cornea	Ohio	FOSC	3.0 (OC-a)
43734	CDC 2006743625	2006	Cornea	New York	FOSC	2.9 (OC-a)
43735	CDC 2006743626	2006	Cornea	New York	FOSC	3.1 (OC-a)
43736	CDC 2006013784	2006	Cornea	California	FSSC 1	11.8 (SC-d), 7.0 (S-1)
43803	AFR4	2006	Contact lens case	Florida	FSSC 1	12.0 (SC-d), 6.8 (S-1)
43804	AFR9	2006	Contact lens case	Georgia	FOSC	3.4 (OC-a)
43805	AFR12	2006	Contact lens case	Georgia	FSSC 1	10.2 (SC-d), 8.3 (S-1)
43806	AFR81036	1981	Cornea	Georgia	FSSC 4	8.8 (SC-d), 7.6 (S-4)
43807	CDC 2006014156	2006	Contact lens solution	Nevada	FOSC	5.1 (OC-a)
43808	CDC 2006743699	2006	Unknown	Texas	FOSC	6.9 (OC-a)
43809	CDC 2006743700	2006	Cornea	Ohio	FSSC 1	14.1 (SC-d), 11.0 (S-1)
43810	CDC 2006743701	2006	Cornea	Ohio	FOSC	7.2 (OC-a)
43811	CDC 2006743702	2006	Cornea	Ohio	FSSC	11.0 (SC-d)
43812	CDC 2006743705	2006	Contact lens solution	New York	FSSC 1	11.2 (SC-d), 9.9 (S-1)
43813	CDC 2006743707	2006	Contact lens case	New York	FSSC 1	15.9 (SC-d), 11.4 (S-1)
43814	CDC 2006743708	2006	Contact lens	New York	FSSC 2	4.6 (SC-d), 9.9 (S-2)
43815	CDC 2006743711	2006	Contact lens case	New York	FSSC 1	10.0 (SC-d), 7.1 (S-1)
43816	CDC 2006743713	2006	Contact lens case	New York	FSSC 1	8.4 (SC-d), 6.2 (S-1)
43817	CDC 2006743714	2006	Contact lens	New York	FSSC 1	13.5 (SC-d), 10.2 (S-1)
43818	CDC 2006743716	2006	Contact lens case	New York	FSSC 2	11.6 (SC-d), 24.0 (S-2)
43819	CDC 2006743718	2006	Cornea	Illinois	FSSC 1	14.5 (SC-d), 11.0 (S-1)
43873	CDC 2006743709	2006	Contact lens case	New York	FSSC 1	10.1 (SC-d), 9.5 (S-1)
43874	CDC 2006743710	2006	Contact lens case	New York	FSSC 2	8.2 (SC-d), 20.7 (S-2)
43875	CDC 2006743712	2006	Contact lens case	New York	FSSC 1+FSSC 2/FSSC 1	18.8 (SC-d), 17.5 (S-1), 5.1 (S-2)
43876	CDC 2006743715	2006	Contact lens case	New York	FSSC 2	8.8 (SC-d), 16.8 (S-2)
43877	CDC 2006743727	2006	Cornea	Tennessee	FSSC 1	9.9 (SC-d), 9.9 (S-1)

<sup>a</sup> AFR, Donald G. Ahearn, Georgia State University, Atlanta, GA; CBS, Centraalbureau voor Schimmelfcultures, Utrecht, The Netherlands; CDC, Centers for Disease Control and Prevention, Atlanta, GA; UTHSC, University of Texas Health Sciences Center, San Antonio, TX.

<sup>b</sup> The four most common species with in the FSSC, designated groups 1 to 4 (5, 44), are indicated.

<sup>c</sup> ID, index of discrimination determined as minimum target fluorescent intensity/maximum non-target fluorescent intensity.

<sup>d</sup> See Table 4 for a complete listing of the probes. Note that the probes listed here lack the F for *Fusarium* prefix.

tag anneals to the complementary anti-tag attached to a unique Luminex xMAP fluorescent polystyrene microsphere, thereby allowing each allele to be scored by flow cytometry. In addition to the panel of 72 phylogenetically diverse fusaria for the design and validation of the microsphere array (DP) (Table 2), an EP comprising 157 medically important fusaria (Table 3) was genotyped by using the microsphere array and also independently by *RPB2* sequence analysis (Table 3). In addition to including 69 CDC keratitis outbreak investigation isolates (5), the EP included 88 clinical isolates from several sources to further test the accuracy of the microsphere array (Table 3).

**PCR amplification for multiplex primer extension.** *RPB2* template for the multiplex primer extension was obtained using the PCR conditions outlined above, with the exception that the 5F2-7cR and 7cF-11aR *RPB2* amplicons of each isolate were pooled at the Montage<sub>96</sub> filter plate purification step. Multiplex primer extension reactions were performed in a total volume of 20  $\mu$ l that included the following: 1 $\times$  PCR buffer, 1.25 mM MgCl<sub>2</sub>, 5  $\mu$ M dATP/dGTP/dTTP, 5  $\mu$ M biotin-dCTP, 25 nM concentrations of each probe primer, 0.75 U of Platinum GenoTYPE *Tsp* DNA polymerase (Invitrogen Life Technologies), and 5  $\mu$ l of purified *RPB2* amplicon. Cycling parameters for the multiplex primer extension reactions included 120 s at 96°C, followed by 40 cycles of 30 s at 94°C, 60 s at 55°C, and 120 s at 74°C, followed by a 4°C soak.

**Hybridization and detection.** Hybridization reactions were conducted in a total volume of 50  $\mu$ l in 1 $\times$  TM buffer (0.1 M Tris, 0.2 M NaCl, 0.08% Triton X-100; Sigma Chemical Co., St. Louis, MO), which included 5  $\mu$ l of multiplex primer extension product, and ~1,250 microspheres of each of the 34 bead sets. Hybridizations were conducted in a MJ PTC-100 thermocycler (MJ Research, Waltham, MA), using the following cycling parameters: 1 cycle of 90 s at 96°C and 45 min at 37°C, followed by a 4°C soak. Hybridization reactions were purified as described previously (8), after which they were resuspended in 70  $\mu$ l of 1 $\times$  TM buffer containing 2  $\mu$ g of streptavidin-R-phycoerythrin conjugate (Invitrogen Molecular Probes, Eugene, OR/ml). Reactions were incubated for 10 min at 37°C in a Luminex 100 flow cytometer, and then the samples were sorted and scored by measuring the fluorescence intensity of biotinylated extension products conjugated to 100 microspheres of each of the 34 probes. Each individual microsphere set used in the assay was labeled with a specific mixture of fluorescent

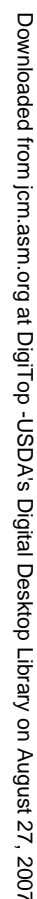
dyes (Luminex Corp.), thereby creating a unique spectral address that enabled the extension products from the 34 different allele-specific probes to be sorted and evaluated individually. Indices of discrimination (ID) (8) for the microsphere array-based identifications were determined by dividing the minimum target fluorescence intensity by the maximum nontarget fluorescence intensity based on duplicate independent runs (Tables 2 and 3).

**Validation of the Luminex assay using a DP.** Duplicate microsphere array-based identifications of all 72 fusaria in the DP, including 36 strains from the CDC's keratitis outbreak investigation (5), were compared to independent *RPB2* sequence-based identifications to assess the accuracy and reproducibility of the microsphere array (Table 2).

**Nucleotide sequence accession numbers.** All DNA sequence data generated in the present study has been deposited in GenBank under accession numbers EF452919 to EF453221 and EF469958 to EF470237. *RPB2* sequences previously published (5) are available under GenBank accession numbers DQ790471 to DQ790602.

## RESULTS

***Fusarium* phylogeny.** Neighbor-joining analyses of *RPB2* sequences of all 318 fusaria included in the present study were conducted to provide an initial assessment of how the keratitis outbreak isolates fell within the full phylogenetic spectrum of *Fusarium*. The results of these analyses were used to construct a DP data set that included representatives of all medically important species complexes and the most important species. The 73 aligned *RPB2* sequences (1,809 bp) of the DP were analyzed by maximum parsimony to assess the phylogenetic diversity of medically and veterinary important fusaria. Thirty-six of the isolates were associated with the contact lens solution keratitis outbreaks of 2005 and 2006 in the United States,



tree, as indicated by bootstrap values ranging from 88 to 100% for the six human pathogen containing species complexes (each indicated by a bold internode in Fig. 1), and ten of the most important species. The root of the outgroup species connected to the tree with the *F. dimerum* (FDSC, 88% bootstrap) and *F. solani* (FSSC, 100% bootstrap) species complexes always forming the basal most branches within the phylogeny.



TABLE 4. Allele-specific primers used in genotyping assay

Target group	Probe <sup>a</sup>	Luminex microsphere <sup>c</sup>	Sequence (5'–3') <sup>b</sup>	
			Luminex probe tag <sup>c</sup>	Probe <sup>d</sup>
Positive control for PCR amplification (5F2 × 7cR)	5f2-aa	35	CAATTTTCATCATTCATTCATTTCA	GGGYCARGCTTGTGGTYTGG
Positive control for PCR amplification (5F2 × 7cR)	5f2-ab	37	CTTTTCATCTTTTCATCTTTCAAT	CARGCTTGTGGTYTGGTC
Positive control for PCR amplification (7cF × 1laR)	7cf-aa	55	TATATACACTTCTCAATAACTAAC	ATGGARTTCCTCAARTTCC
Positive control for PCR amplification (7cF × 1laR)	7cf-ab	78	CTATCTATCTAACTATCTATATCA	ATGGARTTCCTCAARTTCCGTG
FCSC	CC-c	7	CAATTCATTTACCAATTTACCAAT	TTCAGCAGAGAGGGTTTGACC
FCSC	CC-d	44	TCATTTACCAATCTTCTTTTATAC	ACCGTCGATTCCGTYTCGGAACCTC
<i>Fusarium</i> sp. cf. <i>dimerum</i>	D-b	23	TTCAATCATTCAAATCTCAACTTT	TGTTTCAGGATGCCAAGCAATTCTG
<i>Fusarium</i> sp. cf. <i>dimerum</i>	D-c	51	TCATTTCAATCAATCATCAACAAT	AAGCAATTCTGTAATTCGGTAGTC
FDSC	DC-a	59	TCATCAATCAATCTTTTCACTTT	AGGCTTGTGGTYTGGTCAAGAACC
FDSC	DC-d	9	TAATCTTCTATATCAACATCTTAC	GAGTGTCTTCTYAGCAAAGTCTCC
<i>Fusarium fujikuroi</i>	F-b	3	TACACTTTATCAAATCTTACAATC	ACCAAGGGACAAGTAGTGTGACG
<i>Fusarium fujikuroi</i>	F-c	77	CAATTAACATACATACAATACATAC	ACACTCACAGAAGAAGAGAAACGA
<i>Fusarium fujikuroi</i> + <i>F. proliferatum</i>	PF-a	18	TCAAAATCTCAAATACTCAAATCA	GAGGTCATGTACAATGGTCACACG
<i>Fusarium fujikuroi</i> + <i>F. proliferatum</i>	PF-b	30	TTACCTTTTATACCTTTCTTTTAC	GATGTCACAGTCGATTCACTCTCT
<i>Fusarium</i> sp. cf. <i>incarnatum</i>	I-a	26	TTACTCAAATCTACACTTTTTCAT	TGTGAGATTTCATCCCAGTATGATC
<i>Fusarium</i> sp. cf. <i>incarnatum</i>	I-b	10	ATCATACATACATACAAATCTACA	CCTGCCGAGACACCAGAAGGC
FIESC	IEC-b	17	CTTTAATCCTTTATCACTTTATCA	TTCGTCACCGAAGTTGGGTCGGC
FIESC	IEC-f	5	CAATTCAAATCACAATAATCAATC	AAGCATGGAACATACGACAAGCTC
FOSC	OC-a	69	CTATAAACATATTACATTCACATC	AACTACACTGAGATCTTYGAGAAA
<i>Fusarium proliferatum</i>	P-a	28	CTACAAACAAACAAACATTATCAA	GAAGAGGAGAAACGGGCCAAGGCT
FSSC group 1	S-1	40	CTTTCTACATTATTACACAACATTA	GGTGATGCCACGCCCTTTCACCGAC
FSSC group 2	S-2	94	CTTTCTATCTTCTACTCAATAAT	GGTATCGTGGCYCCCGGTGTGCGC
FSSC groups 3 and 4	S-34	14	CTACTATACATCTTACTATACTTT	CGCTTGCTACTCTGGTTACAACCAA
FSSC group 4	S-4	6	TCAACAATCTTTTACAATCAAATC	ACGCCGCTGCGAAGTACCGAGAAT
FSSC	SC-d	95	TACACTTTAACTTACTACACTAA	ACAATTGCMCATTGTATTGAATGC
FSSC	SC-f	76	AATCTAACAACTCATCTAAATAC	ATTGAATGCCTCCTCAGTAAGGTG
<i>Fusarium thapsinum</i>	T-b	29	AATCTTACTACAAATCTTCTTTT	ATGTGTTATGTCAAGTGTGCGCTCC
<i>Fusarium thapsinum</i>	T-e	100	CTATCTTTAACTACAAATCTAAC	CAAGTCATCTGACAGTCAACGCG
<i>Fusarium verticillioides</i>	V-a	33	TCAATTACTTCACTTTAATCTTTT	CTTGTCGTGATATCCGAGACCGC
<i>Fusarium verticillioides</i>	V-d	12	TACACTTTCTTTCTTTCTTTCTTT	CTTGATGAGGATGGTATCGTGGCA
GFSC	GFC-a	65	CTTTTCATCAATAATCTTACCTTT	GTYCACTTCGAAGTATCRCTTGT
GFSC	GFC-c	85	ATACTACATACATAATCAAACATCA	CRGAAGARGAGAARCGRGCYAAGG
GFSC + FOSC	GFCO-c	80	CTAACTAACAAATAATCTAACTAAC	YCGTGARCARAAGAATGATGARGC
GFSC + FOSC	GFCO-d	24	TCAATTACCTTTTCAATACAATAC	GACAGGAGGATATGCCTTTCAGCC

<sup>a</sup> Minimum fluorescence intensity values for the 12 probes in boldface were not used to calculate the ID, because they did not perform as well as the other probes. The V-d probe frequently gave high background non-target fluorescence values and was therefore excluded from the ID scores.

<sup>b</sup> M, AC; R, AG; Y, CT.

<sup>c</sup> Microspheres and tag sequences were assigned at the Luminex website.

<sup>d</sup> Probe sequences used to identify the various target groups within *Fusarium* spp.

The branching order of the FDSC and FSSC, however, was unresolved (bootstrap 55%). Phylogenetic relationships of the most important human pathogenic members of the species-rich FSSC were nearly completely resolved (Fig. 1), as reflected by bootstrap values of 90 to 100% for the four most important species groups (i.e., groups 1 to 4 [44]). The remaining ingroup taxa, comprising the *Gibberella* clade, were strongly supported as monophyletic (98% bootstrap). Phylogenetic results support a sister group relationship between the *F. chlamydosporum* (FCSC, 100% bootstrap) and *F. incarnatum-equiseti* species complexes (FIESC, 100% bootstrap), and the terminal sister groups, the *Gibberella fujikuroi* (GFSC, 97% bootstrap) and *F. oxysporum* species complexes (FOSC, 100% bootstrap).

**Multilocus DNA sequence-based genotyping.** All 191 CDC keratitis investigation isolates from cornea or patient environ-

ments were genotyped by using multilocus DNA sequence typing (MLST) (5) (Table 1 and Table S1 in the supplemental material). Included among these were replicate isolates from the cornea of 15 separate patients and from the environments of 14 separate patients, which appeared to be putative clones based on the MLST data (Table 1 and Table S1 in the supplemental material). Although 13 MLSTs were represented, three haplotypes accounted for about two-thirds of the putative clones (i.e., FSSC 1-a, FSSC 2-d, and FOSC 3-a; Fig. 1). Of the 10 matched corneal-environment isolates, 6 were from confirmed cases, and each of the 10 paired isolates shared the same MLST. In addition, all of the replicate isolates from each patient's environment ( $n = 14$ ) shared the same MLST (see Table S1 in the supplemental material). In all, the corneal isolates comprised 29 multilocus haplotypes distributed among 16 species, whereas the environmental isolates included 27

haplotypes and 15 species (Table 1). Although isolates from five species complexes were represented among the corneal isolates (Table 1), members of the FSSC comprised 61.8% of the isolates. FSSC haplotypes 1-a and 2-d, representing two phylogenetically distinct species, accounted for the majority of FSSC isolates from patients and their environment. Collectively, the FSSC was represented by 24 multilocus haplotypes, including 14 from corneal isolates and 14 from the environmental assessment, and these were distributed among nine phylogenetically distinct species (i.e., FSSC 1 to 9). The only other complex represented by significant numbers was the FOSC, which accounted for 29.7% of the corneal and 29% of the environmental isolates (Table 1). Overall, the FOSC was represented by 10 unique multilocus haplotypes, with the widespread FOSC 3-a clonal lineage (33) accounting for the majority of all isolates within this complex. The single most common haplotype from each of the three most common species (i.e., FSSC 1-a, FSSC 2-d, and FOSC 3-a) collectively comprised 55% of the corneal and environmental isolates (Table 1 and Table S1 in the supplemental material). Moreover, members of the FSSC and FOSC accounted for 91.1% of the CDC keratitis outbreak isolates and 34 of the 45 multilocus haplotypes. The 17 remaining outbreak investigation isolates were distributed among three species complexes (Table 1; GFSC, FIESC, and FDSC) and 11 phylogenetically distinct species; however, they accounted for only 11% of the corneal and 7% of the environmental isolates.

**Allele-specific genotyping.** Independent *RPB2*-based identifications of the 72 isolates in the DP revealed that all were correctly identified to species and/or species complex by the microsphere array (Table 2). ID values for the species and/or species complex probes for the 72 DP isolates ranged from 2.6 to 53.2 with a mean fluorescence of 10.1. Moreover, 96 of 101 positives in the DP had fluorescence intensity values of at least three times above background (95%; Table 2). Of the 34 probes, 23 accounted for 100% of the ID scores for the DP (Table 2) and the EP (see below; Table 3). However, only the ID scores for the *F. verticillioides* V-d probe were excluded from the analysis because it frequently gave false-positive results.

An EP consisting of 157 clinical isolates was genotyped by using the validated microsphere array and also independently by *RPB2* sequence analysis (Table 3). The EP included 69 CDC keratitis outbreak investigation isolates (5), 27 additional clinical isolates from the CDC unrelated to the keratitis investigation, 54 clinical isolates from the Fungus Testing Laboratory at the University of Texas, San Antonio, together with 7 isolates from other sources (Table 3). All 69 CDC keratitis outbreak isolates genotyped by the microsphere array were correctly assigned to species and/or species complex (Tables 2 and 3), as confirmed by the independent *RPB2* sequence analysis. Of the 105 CDC keratitis outbreak isolates genotyped in the combined DP and EP, 87.5% of the FSSC (56 of 64) and all 8 GFSC isolates were identified to species by using the single locus assay. The eight isolates for which species identity could not be determined using the microsphere assay were all correctly assigned to the FSSC. However, the *RPB2* and MLST data (Table 1 and Table S1 in the supplemental material) indicated that they represented relatively uncommon taxa that

were not included in the DP. Therefore, probes for these rare species were not represented in the microsphere array.

All of the EP isolates were accurately assigned to one of six species complexes by the microsphere array, as confirmed by the *RPB2* sequence data (Table 3), with the exception of NRRL 13459 *F. concolor* (later synonym = *F. polyphialidicum*) and NRRL 43641 *Fusarium* sp. (ex horse eye), each of which represents an additional evolutionary lineage within *Fusarium*. However, in contrast to the DP test results, in which all of the isolates were correctly assigned to species and/or species complex, nine EP isolates not involved in the keratitis outbreaks were misidentified at the species level by the microsphere array (i.e., 94.3% accuracy). Seven of the nine misidentifications involved CDC isolates that were incorrectly identified as FSSC group 1 (NRRL 28008 to 28011) or FSSC group 3 (NRRL 28000 to 28002) by the S-1 and S-34 probes, respectively. Analysis of the *RPB2* sequence data indicated that the FSSC isolates that were misidentified represent three species that are phylogenetically distinct from FSSC groups 1 to 4 (44). It is also noteworthy that the *F. proliferatum*-*F. fujikuroi* PF-b group probe incorrectly indicated that *F. concolor* was a member of the GFSC. However, the GFSC-specific GFC-c probe for this species complex was negative, indicating the result obtained with the PF-b group probe was in error. Subsequently, this finding was confirmed independently by *RPB2* sequence analysis. The only other isolate misidentified at the species level by the microsphere array was FDSC isolate NRRL 43716 *Fusarium* sp. (ex human blood), which was typed as *F. sp.* (cf. *F. dimerum*) (*F. sp.* cf. *dimerum*) with the D-b and D-c species probes. Although the *RPB2* sequence data determined this species identification was incorrect, the microsphere array correctly assigned this isolate to the FDSC (Table 3). We determined that all nine species level misidentifications were not false positives by comparing the five probe sequences with the respective nontarget sequence. These comparisons revealed that each probe sequence was either an exact match (S-34, D-b, and D-c) or differed by a single nucleotide in the middle of the probe (S-1 and PF-b).

ID values for the target species and/or species complex probes for the 157 EP isolates ranged from 2.6 to 47.1, with a mean of 10.9. Also, 245 of the 250 positives in the EP had fluorescence intensity values at least three times above background (98%; Table 3). In the combined DP and EP data, 341 of the 351 positives had fluorescence intensity values at least three times above background (97.2%; Table 3), with a mean ID of 10.7. Lastly, NRRL 43875 (CDC 2006743712 ex contact lens case in Kentucky) was identified as FSSC group 1 by the *RPB2* sequence data; however, the microsphere array indicated that it was a mixed culture containing FSSC groups 1 and 2.

## DISCUSSION

**Multilocus and allele-specific genotyping.** The present study extends CDC's keratitis outbreak investigation that established use of the since discontinued Bausch & Lomb's ReNu with MoistureLoc contact lens solution as a significant risk factor for acquiring FK among soft contact lens wearers (5). Moreover, we provide here the first detailed genotypic comparison of the fusaria causing keratitis with those recovered from pa-

tient's environments (i.e., contact lens and lens case). In addition to facilitating an early diagnosis of FK and prompt antifungal therapy (4), the availability of cultures from corneal scrapings and environmental isolates played a central role in developing objective epidemiological data and molecular diagnostic tools in the present study.

In contrast to a phenotype-based report that members of the FOSC were the most common fusaria associated with the outbreaks in 2005 and 2006 (1), the molecular phylogenetic data presented herein and by Chang et al. (5) clearly demonstrate that FSSC outbreak isolates from the United States (including Puerto Rico) collectively accounted for twice as many corneal isolates as those from the FOSC (Table 1). When this comparison was restricted to the keratitis outbreak isolates from Florida (15 corneal and 6 environmental; see Table S1 in the supplemental material), all of these isolates were identified as members of the FSSC, except for two isolates of the widespread FOSC 3-a clonal lineage (33). Similar findings that all of the outbreak isolates of 2005 and 2006 from Singapore and Hong Kong were members of the FSSC (5, 20) are consistent with the results of previous studies that have documented the predominance of FSSC fusaria in causing ocular mycoses worldwide (13, 26, 44). The results of Chang et al. (5) and the present study, however, have established that phylogenetically diverse members of the FOSC can infect the human cornea (Table 1). This finding is one of the surprising results to emerge from the present study, in that the only comprehensive molecular phylogenetic analysis of medically important FOSC concluded that members of this complex might only rarely cause eye infections (33). Another surprise was our finding that corneal infections were most frequently associated with FSSC group 1 and least frequently associated with FSSC group 3, whereas Zhang et al. (44) reported the converse. To reconcile these seemingly conflicting reports, Chang et al. (5) hypothesized that FSSC group 3 isolates may be most commonly associated with ocular trauma, given they are commonly found in soil and on vegetation; however, patients with a recent history of ocular trauma were excluded from the keratitis outbreak investigation by CDC's case definition. It is also noteworthy that the initial report by Chang et al. (5) and the present study are the first to conclusively document *F. fujikuroi* as a human pathogen, and these involved four separate patients with infections of either their cornea, skin, or nails (Tables 2 and 3).

Our finding that the three haplotypes most frequently recovered from the patient's environment (FSSC 1-a, FSSC 2-d, and FOSC 3-a) were also responsible for the greatest numbers of ocular infections strongly suggests, as originally proposed by Chang et al. (5), that the majority of the keratitis outbreak infections were likely caused by the most prevalent fusaria in the patient's environment at the point of contact lens and lens solution use. This conclusion is supported by the fact that each of the 10 matched corneal-environment isolates we genotyped shared the same MLST, suggesting that extrinsic contamination of the patient's contact lens and lens cases played a major role in the keratitis outbreaks. The high-degree of matching between corneal and environmental isolates also supports the common ophthalmologic practice of culturing contact lens supplies when contact lens-associated corneal infection is suspected (7). By way of contrast, *Fusarium* was not recovered by the CDC from unopened contact lens solution bottles from

relevant lots of ReNu with MoistureLoc nor from environmental samples taken from Bausch & Lomb's production facility in Greenville, SC, providing additional evidence that the outbreaks were not due to a common point-source (5). Although genetically matching isolates were obtained from contact lens and bottles of opened MoistureLoc lens solution from patients in Georgia (FOSC 3-a) and Pennsylvania (FSSC 3-a) in the present study (see Table S1 in the supplemental material) and from a patient's lens case and opened bottle of MoistureLoc in New York state (9), all of the available evidence supports the conclusion that unopened bottles of ReNu with MoistureLoc are sterile microbiologically. We hypothesize that the patient's water system (e.g., bathroom sink drain) may represent the primary reservoir of infection, largely because the three predominant haplotypes we recovered from cornea and the patient's environment appear to be common and widespread inhabitants of sink and shower drains (33, 44), including those in hospitals where they may potentially contribute to life-threatening nosocomial infections among immunocompromised patients (2, 22). Assuming that the three prevalent haplotypes are particularly well adapted to water systems, such as sink drains, it seems probable that any shared adaptations predate the evolution of these phylogenetic lineages, given that these haplotypes exhibit a polyphyletic distribution in the *Fusarium* phylogeny (Fig. 1).

Reproducibility of the *Fusarium* microsphere array was confirmed in the present study by duplicate runs of all 229 isolates we typed. Minimum fluorescence scores for isolates with a positive genotype were always at least two and one-half times greater than the maximum fluorescent intensity scores for those with a negative genotype. However, based on independent comparisons of the microsphere array-based identifications with those obtained via *RPB2* sequencing and/or MLST, the current array has several noteworthy limitations that need to be addressed before clinical laboratories can benefit from its full potential. The present allele-specific genotyping scheme, which is based exclusively on *RPB2* nucleotide variation, incorrectly identified a small number of isolates at the species level within the species-rich FSSC (Table 3), and it lacked the power to resolve the multilocus genotypes identified by MLST within the FSSC, FIESC, and FOSC (Table 1 and Table S1 in the supplemental material). Because the current assay only detects a relatively small number of *RPB2* genotypes, ongoing efforts are directed at expanding the database to facilitate MLST-based estimation of haplotype-disease associations in the future.

***Fusarium* phylogeny.** The present study extends our growing knowledge of the evolutionary relationships and phylogenetic diversity of medically and veterinary important fusaria, focusing on isolates from the keratitis outbreaks of 2005 and 2006 associated with the use of ReNu with MoistureLoc contact lens solution within the United States (5). Prior to the present study, published phylogenies that spanned the breadth of the genus were based exclusively on analyses of domains D1+D2 of the nuclear large subunit (LSU) ribosomal DNA (rDNA) (17, 39), primarily because this region is conserved enough such that a genus-wide alignment reflects positional homology and secondarily because of the ready availability of diverse fusarial LSU rDNA sequences from GenBank. However, because the LSU molecule is so highly conserved and the region



sequenced in these studies is so small (440 to 500 bp), it is not surprising that this locus contains relatively little phylogenetic signal within *Fusarium*. As a result, the bootstrap values recovered were much too low for the LSU-based phylogenies to represent meaningful hypotheses of evolutionary relationships within *Fusarium*. For example, the FOSC, FSSC, and GFSC received bootstrap support of 68%, 35 to 67%, and 56 to 79%, respectively, in the two aforementioned LSU rDNA-based phylogenies. By way of contrast, the monophyly of these three clades was strongly supported in the present *RPB2* phylogeny as reflected by bootstrap values of 97 to 100% (Fig. 1). Not surprisingly, the multilocus species level studies published to date, that included nuclear LSU and/or ITS rDNA sequence data, clearly demonstrate that molecular diagnostic assays targeting either the LSU (15, 28) or ITS rDNA (18, 43) would fail to differentiate medically important species within several species complexes within *Fusarium*.

Although frequently analyzed as protein coding sequence to help elucidate deep-level evolutionary relationships within the fungi (19, 23), several studies have demonstrated the utility of *RPB2* nucleotide variation at the species level in diverse fungi (10, 16). The present study highlighted two attractive features of *RPB2* nucleotide sequence data for phylogeny reconstruction within *Fusarium*: the region sequenced (1.8 kb) was easily aligned across the entire genus, and the wealth of phylogenetic signal contained within this locus provided strong bootstrap support for many clades for the first time. However, given the poor bootstrap support for the branching order of the two basal-most clades (i.e., FSSC and FDSC), data from additional phylogenetically informative loci will be required to fully resolve the phylogeny for this medically and agriculturally important genus. Nucleotide sequence data from translation elongation factor 1 $\alpha$  have been extraordinarily useful in *Fusarium* phylogenetics within species complexes and at the species level (reference 11 and references therein); however, the large phylogenetically informative intronic regions within this gene are much too variable to align across the genus.

A noteworthy finding to emerge from the present study is that 99.4% of the clinical fusaria genotyped were nested in one of six monophyletic species complexes. Members of two other evolutionary lineages were identified, but they are both represented by a single known keratitis isolate, *Fusarium* sp. strain NRRL 43641 from a horse in Missouri and *F. concolor* from a human corneal ulcer in Spain (14). Although we genotyped several isolates from the FCSC, including human and equine corneal isolates from Florida (NRRL 43632) and Texas (NRRL 43636), respectively, there is only one published report of a member of this species complex causing keratitis (26). No member of this complex, however, was encountered in the present keratitis outbreak investigation. Unfortunately, conventional taxonomic practice has been to treat members of the FSSC, FOSC, FCSC, and FDSC each as a single morphospecies (27; i.e., *F. solani*, *F. oxysporum*, *F. chlamydosporum*, and *F. dimerum*, respectively) and members of the FIESC as only two morphospecies (i.e., *F. incarnatum* and *F. equiseti*; the former morphospecies is also reported as *F. semitectum*). The present study is the first to highlight the need for multilocus species level studies within the FCSC, FDSC, and FIESC, each of which appears to contain multiple morphologically cryptic

species pathogenic to humans based on the present phylogenetic results. Although multilocus phylogenetic hypotheses of evolutionary relationships have been developed for the FSSC (29, 44), FOSC (31, 33), and GFSC (30, 32), additional species level studies that use multilocus phylogenetic species recognition (42) are needed within the pathogen-rich FSSC to clarify the limits of the approximately 18 phylogenetically distinct and medically important species that are nested within clade 3 of this complex (44). Fortunately, there is a growing recognition within the medical community that isolates identified as *F. solani* typically represent multiple genetically diverse species (5, 13, 26). However, at present species names can only be confidently applied to two of the human pathogenic members of the FSSC, and these species were only recently transferred to *Fusarium* from *Acremonium* and *Cylindrocarpon* where they had been misplaced (39).

**Future directions.** Because MLST typing schemes are so labor-intensive and expensive and because they are currently considerably less efficient than allele-specific assays such as the one developed herein, the present study should be viewed as just the first step in the transition from a multilocus DNA sequence-based to a single nucleotide polymorphisms (SNP)-based platform for genotyping medically important fusaria and their epidemiologically relevant haplotypes. It is noteworthy that an SNP-based assay has been developed recently for toxigenic fusaria (21). Because it is relatively easy to design SNP or allele-specific probes from a comprehensive set of aligned, phylogenetically informative DNA sequences, improvements in the present assay could easily take advantage of allelic variation identified in the present and future multilocus phylogenetic analyses. Given the electronic portability of multilocus DNA sequence data (25), future improvements of the *Fusarium*-ID database (11) will also incorporate MLST data on clinically important fusaria to help facilitate strain typing via the internet. However, irrespective of what genotyping platform is used in the future, the utility of the MLST data is expected to increase as the database expands. Ongoing whole-genome sequencing projects of four phylogenetically diverse fusaria (<http://www.broad.mit.edu/annotation/fungi/fgi/> and <http://www.jgi.doe.gov/>) should greatly accelerate the discovery of phylogenetically informative loci and population level molecular markers. Additional multilocus molecular phylogenetic analyses that use dense taxon sampling should prove to be invaluable in improving our understanding of species limits and their reproductive mode and in identifying nucleotide variation necessary for increasing the discriminatory power of the *Fusarium* database so that robust typing and subtyping assignments can be achieved. In addition, pathogen control programs and future outbreak investigations could greatly benefit from microsatellite (41) and variable numbers of tandem repeat-based typing schemes (37) for elucidating the population structure and reproductive mode of the most common and geographically widespread human pathogenic fusarial haplotypes, such as FSSC 1-a, FSSC 2-d, and FOSC 3-a. Such studies will make robust clone or clonal lineage assignments possible for the first time, which are essential for objective epidemiological assessments of their host range, virulence (34), geographic distribution, and population of origin.



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The mention of trade products or firm names does not imply that they are recommended or endorsed by the U.S. Department of Agriculture over similar products or other firms not mentioned. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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